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Localized complement activation in the development of protective immunity against *Ostertagia ostertagi* infections in cattle

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ABSTRACT

The abomasal nematode *Ostertagia ostertagi* is a major causal agent contributing to production inefficiencies in the cattle industry in temperate regions of the world. Protective immunity to infections develops very slowly and resistance to reinfection manifests only after prolonged exposure. Mechanisms underlying the development of protective immunity remain largely unexplored. Immune animals, which have significantly reduced worm burdens, were developed after multiple drug-attenuated experimental infections and were compared to a primary infected group and their respective uninfected controls. In this study, transcriptomic analysis identified three signaling pathways significantly impacted during both primary and repeat infections, the complement system, leukocyte extravasation and acute phase responses. Increased mRNA levels of complement components C3, factor B (CFB) and factor I (CFI) in the abomasal mucosa of the infected cattle were confirmed using quantitative PCR while Western blot analysis established the presence of elevated levels of activated C3 proteins in the mucosa. One of the initiators of local complement activation could be related to secretory IgA and IgM because infections significantly up-regulated expression of J chain (IGJ), as well as polymeric Ig receptor (PIGR) and an IgM-specific receptor (FAIM3), suggesting sustained increases in both synthesis and transepithelial transport of IgA and IgM during the infection. The elevated levels of pro-inflammatory cytokines, such as IL-4 and IL-1β, during infection may be involved in gene regulation of complement components. Our results suggest enhanced tissue repair and mucin secretion in immune animals may also contribute to protective immunity. These results are the first evidence that local complement activation may be involved in the development of long-term protective immunity and provide a novel mechanistic insight into resistance against *O. ostertagi* in cattle.

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1. Introduction

The abomasal nematode *Ostertagia ostertagi* is a major causal agent contributing to production inefficiency in cattle industry in temperate regions of the world. One of the major patho-physiological changes associated with ostertagiosis in cattle is impaired gastrointestinal (GI) function, including reduced gut motility and decreased gastric acid secretion (Fox, 1997). Ingested larvae cause considerable tissue damage to the abomasal mucosa, including hyperplasia of gastric glands, severe epithelial cytolysis, and loss of acid-producing parietal cells, resulting in elevated abomasal pH levels and impaired protein metabolism. Clinical manifestations of moderate to severe *Ostertagia* infections in young calves may include diarrhea, malnutrition and even death.

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It has long been recognized that protective immunity to *O. ostertagi* infection, as defined as resistance to the establishment of worms, in cattle is very weak and develops very slowly, requiring prolonged exposure before immunity becomes effective (Michel et al., 1973; Gasbarre, 1997). This is in sharp contrast to other cattle parasites, such as *Dictyocaulus viviparus* or *Oesophagostomum radiatum*, where resistance develops quickly and the host becomes refractory to reinfection. An effective protective immune response usually becomes evident when cattle approach 18 months of age, after grazing on pastures naturally infected with the parasite. Even in cattle of this age there are low or even considerable burdens of *Ostertagia* (Armour, 1989). Experimentally, three drug-attenuated infections provide only partial immunity against experimental challenge, leading to about a 43% reduction in worm burdens (Almeria et al., 1998). It can require five drug-attenuated infections with *O. ostertagi* to see a significant reduction in mean worm counts (up to 73%) when compared to a primary infection. In addition, a large burden of inhibited early L4 larvae can exist in the fall and winter in cattle that are exposed to infection during spring and summer (Michel et al., 1973). This prolonged susceptibility to reinfection is a major reason why this parasite remains the most economically important GI nematode in temperate climate areas of the world (Gasbarre, 1997).

Host immune responses to *O. ostertagi* infection in cattle have been examined. *Ostertagia* antigens are readily presentable to the host in draining lymph nodes shortly after infection. It becomes evident that the size and cell population profiles of regional lymph nodes undergo drastic changes (Gasbarre, 1986). A concomitant increase in the number of lymphocytes in the abomasal mucosa is observed 3–4 days after infection. The infection is able to induce expression changes in a number of cytokines in both abomasal lymph nodes and in abomasal mucosa. In lymphocytes isolated from abomasal lamina propria, expression levels of IL-4 and interferon gamma (IFNG) are up-regulated by a primary *O. ostertagi* infection at both 10 days post-infection (dpi) and 60 dpi (Almeria et al., 1997). Cell population profiles of local lymph nodes of immune animals after five drug-attenuated infections also show changes compared to cattle with only a primary infection. The most notable is higher numbers of $CD4^+/CD8^+$ cells and decreased levels of IgM^+ (B) cells in immune animals (Almeria et al., 1998). Accompanying these changes in cell populations in lymph nodes is a shift in cytokine profiles. For example, the mRNA level of IL-4 was significantly decreased in immune animals compared to primary infected animals. While mRNA levels are significantly correlated with the number of worms, IL-4 may not be associated with any protective mechanism in the cattle–*Ostertagia* system (Almeria et al., 1998). The role of other cytokines in the development of protective immunity remains unclear. No correlation is generally observed between cytokine transcription levels and protection (Claerebout et al., 2005). Possible reasons for the slow development of protective immunity against *Ostertagia* have been postulated. Among these possible mechanisms is the ability of *Ostertagia* to suppress host immune responses (Klesius et al., 1984; Gomez-Munoz et al., 2004), and induce a transient reduction in host immune reactivity (Gasbarre, 1997). Understanding mechanisms of protective immunity has both practical and theoretical implications. For example, despite continuous efforts to identify protective antigens, efficacious vaccines that render protection, i.e., resulting in a reduction in worm burdens, have yet to be developed. This is mainly due to limited knowledge of protective immune responses and a lack of understanding of specific immunological parameters for acquired immunity against *Ostertagia* (Claerebout and Vercruysse, 2000). Molecular characteristics of *Ostertagia* antigens able to elicit strong protective immune responses remain unknown, hindering development of effective antigen delivery systems during vaccine development. The magnitude and mechanisms of immunosuppression induced by *Ostertagia* and its impact on animal health have not been defined (Gasbarre, 1997). Readily discernable markers for protective immunity would allow farmers to utilize host immunity as part of their management practice. In this study, we attempt to gain further insight into the mechanisms of protective immunity in the cattle–*Ostertagia* system.

2. Materials and methods

2.1. Animals and parasitology

Sixteen Holstein bull calves were purchased locally within 2 days of birth. After weaning, these calves were fed *ad lib* a standard calf ration and maintained on concrete from acquisition through the duration of the experiment. Oral infection with *O. ostertagi* infective L3 larvae (100,000 larvae per calf) was initiated after calves reached 3–4 months of age. The infective L3 larvae were obtained from cultures maintained at USDA-ARS Beltsville facilities. Two types of experimental challenges were conducted. The primary (initial) infection ($N = 4$) was allowed to progress for 14 days. Age-matched, uninfected naïve calves ($N = 4$) were used as controls. For the drug-attenuated (repeat) infection, all calves were initially orally infected with 100,000 L3 infective larvae for 14 days and then treated with a 2× labeled dose of fenbendazole (Safe-Guard) to remove existing parasites. The calves were allowed to rest for 30 days on concrete before a 2nd round of infection with the same number of L3 larvae for 14 days. This infection-drug treatment-resting cycle was repeated four times on all eight calves. During the final drug-attenuated infection (repeat infection), four calves were drug treated and allowed for resting for 3–4 weeks and then orally dosed with a tap water placebo. These calves were used as drug-attenuated controls. The remaining four calves, which also underwent four infection-treatment-resting procedures, were orally infected with 100,000 L3 infective larvae for 14 days. These calves can be considered as immune animals after five drug-attenuated infections. At the end of the experiment, calves were sacrificed; and full thickness folds from the fundic abomasa were collected. The tissue sample was snap frozen in liquid nitrogen prior to storage at −80 °C until total RNA was extracted. The animal maintenance and handling were based on the protocol approved by The USDA-ARS Animal Care and Use Committee; and

Institutional Animal Care and Use Committees guidelines were strictly followed. Fecal egg count (EPG) was monitored during the repeat infection experiment using zinc sulfate double centrifugation and parasite burdens were determined as previously described (Li et al., 2007).

2.2. Quantitative RT-PCR, microarray analysis, and pathway analysis

Total RNA extraction, quantitative (real-time) PCR and microarray fabrication and hybridization were previously described (Li et al., 2006; Li and Gasbarre, 2009). Briefly, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and further purified using an RNeasy Mini kit (Qiagen, Valenica, CA). RNA integrity was verified using a Bioanalyzer 2100 (Agilent, Palo Alto, CA). For real-time RT-PCR, cDNA synthesis was performed using an iScript cDNA Synthesis kit (BioRad, Hercules, CA). Real-time RT-PCR analysis was carried out using an iQ SYBR Green Supermix kit (BioRad) with 200 nM of each amplification primer and first-strand cDNA (100 ng of the input total RNA equivalents) in a 25-μl reaction volume as described (Li et al., 2006). Amplification was carried out on an iCycler iQ Real-Time PCR Detection System (BioRad) with the following profile: 95 °C – 60 s, 40 cycles of 94 °C – 15 s, 60 °C – 30 s, and 72 °C – 30 s and a melting curve analysis was performed for each primer pair. Standards for each gene were prepared from PCR products purified using the DNA Clean and Concentrator kit (Zymo Research, Orange, CA). Expression levels were determined from a standard curve of known target cDNA copy numbers (1.0×10^1 to 1.0×10^6 molecules), which was analyzed simultaneously with the experimental samples.

The bovine microarray, which included 86,191 unique 60mer oligonucleotides synthesized *in situ*, each repeated four times on the microarray, representing 45,383 bovine genes and/or expressed sequence tags (ESTs), was previously described (Li et al., 2006; Li and Gasbarre, 2009). After hybridization, scanning and image acquisition, data were extracted from raw images using NimbleScan software (Roche, Indianapolis, IN). A total of 16 microarrays, 4 biological replicates per treatment group, were used in this experiment (GEO accession# GSE20927). Relative signal intensities (log2) for each feature were generated using the robust multi-array average algorithm (Irizarry et al., 2003). The data were then processed based on the quantile normalization method (Bolstad et al., 2003). The background-adjusted, normalized, and log-transformed intensity values were further analyzed using MeV v4.2 (http://www.tm4.org/mev/). Genes were filtered as suggested by Guo et al. (2006) based on their significance ($P < 0.05$) and followed by fold change (2-fold as a cutoff), resulting in a more consistent gene list for downstream pathway analysis.

Genes significantly regulated during infection were analyzed using Ingenuity Pathways Analysis (IPA) software v7.5 (Ingenuity Systems, Redwood City, CA) as described previously (Li and Capuco, 2008). Briefly, the significant genes with known gene identifiers (gene symbols) and their corresponding expression values were uploaded into the software. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Canonical pathways were identified from the IPA library of canonical pathways based on two parameters: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway, and (2) a *P* value calculated using Fischer's exact test, which determines the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

2.3. Western blot analysis

Crude proteins were extracted from bovine fundic abomasal samples. Ground tissue powder was mixed (1:5, w/v) with Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) with a protease inhibitor cocktail (Sigma, St. Louis, MO) added prior to use. Samples were then homogenized using a Polytron for 20 s at 4 °C and the obtained homogenates were briefly centrifuged at 4 °C for 2 min at $10{,}000 \times g$ to remove debris. Crude protein was quantified using a modified Branford method and Western Blot analysis performed as described (Li et al., 2009). Briefly, the protein from different samples was separated by SDS PAGE on two identical 4–20% polyacrylamide gradient gels. One gel was stained with Simply Blue Safestain (Invitrogen) and served as loading control. Another gel was used for Western blot and imaging analysis. The Western blot was probed with the following primary antibodies, bovine IL-1β and IL-4 polyclonal antibodies (Pierce), human activated complement C3 (I3/15; sc-47687; Santa Cruz Biotechnology, Santa Cruz, CA, USA), human IGLL1 (Lambda 5, sc-28106, Santa Cruz Biotechnology) and human NCF4 (p40-phox, sc-18252, Santa Cruz Biotechnology) antibodies. After washing these blots were incubated with an IRdye labeled secondary antibody (Li-Cor Bioscience, Lincoln, Nebraska). The bands were detected using a Li-Cor Odyssey Infrared Imaging System (Li-Cor). The relative density of the target bands on the blots was quantified using the imaging software UN-SCAN-IT (Silk Scientific, Orem, UT).

3. Results

3.1. Worm burdens

The number of adult worms recovered from the abomasum is summarized in Table 1. The percentage of larvae in total worms recovered after the primary infection was sta-

Table 1
Worm burdens in the bovine abomasums during *Ostertagia ostertagi* infection. Age and worm count data ($N = 4$) were expressed as mean ± SEM.

Treatment	Age (days)	Adult worm[a]	% Larvae[b]
Naïve CT	111 ± 2	0 ± 0	0.00%
Primary infection	115 ± 1	1883 ± 603	1.49%
Drug-attenuated CT	258 ± 2	50 ± 44	0.00%
Repeated (5th) infection	258 ± 2	3845 ± 1564	13.22%*

[a] Infectivity of *Ostertagia* L3 between the two different infections varied greatly.
[b] The percentage of larvae in total worms recovered.
* $P < 0.05$.

tistically significant ($P = 0.024$) at 1.49% compared to 13.22% in the re-infected group, suggesting that a strong protective immunity was indeed developed in these animals. This observation was consistent with an earlier published report which stated that previous infections tended to increase the proportion of larvae (Michel et al., 1973). In contrast to the naïve control, in which no worms were recovered, an average of 37.5 worms was recovered from the drug-attenuated control group. Infectivity of *Ostertagia* L3 used in both primary and drug-attenuated infections varied greatly. Therefore, a direct comparison of worm burdens from these infections should be avoided. A significant reduction in worm counts (up to 70%) after five drug-attenuated infections using the same *Ostertagia* strain is frequently achieved in our previous experiments.

3.2. Genes and pathways significantly impacted by *Ostertagia* infection

Twenty-two genes were significantly impacted in the fundus region of the abomasum during the primary infection compared to the age-matched naïve controls. Among these genes, only common salivary protein BSP30, form b (SPLUNC2B) and a transcript similar to methyltransferase like 7A (METTL7A) were down-regulated. Two hundred sixteen unique sequences were significantly impacted in the animals after the repeat infection compared to their drug-attenuated controls. However, only a small portion of these sequences had known function (Table 2).

Sixteen genes were significantly up-regulated during both the primary and repeat infections compared to their respective controls. The most notable were trefoil factor 3 (intestinal) (TFF3), chloride channel, calcium activated, family member 1 (CLCA1), claudin 2 (CLDN2), tenascin C (TNC), microseminoprotein, beta-(MSMB) and Fc fragment of IgG binding protein (FCGBP). Several matrix metallopeptidases (MMP), including MMP1, MMP3 and MMP13, were also strongly up-regulated during both infections.

There were several types of lectins strongly up-regulated in the abomasal mucosa during infection, including the extracellular lectins (C-type, such as collectin-46) and galectins, such as galectin-15, LGALS15, as well as intelectin 2 (ITLN2) from a novel group. Another bovine-specific collectin, conglutinin, was also ~3-fold higher during reinfection compared to the drug-attenuated controls, however the difference was not statistically significantly. Up-regulation of TFF3 in both the primary and repeat infections in cattle may represent a response to general gastric damage or inflammation. Enterophilin-like gene, a molecule with a restricted expression in enterocytes and involved in cell differentiation in gut epithelia, and gastrokine 2 (GSK2), which may modulate gut epithelial cell proliferation, were down-regulated during infection.

Pathway analysis identified three canonical pathways, the complement system, acute phase response signaling and leukocyte extravasation, significantly ($P < 0.05$) impacted during infection (Fig. 1). Three complement components, C3, factor B (CFB) and factor I (CFI), one of the key regulators of the alternative pathway of the complement cascade, were strongly up-regulated locally in the

Table 2
Genes significantly induced in the bovine fundic abomasum during *Ostertagia ostertagi* infection.

Oligo ID	Gene symbol	Primary infection	Repeat infection
BOVINE0001S00019162	ABAT	0.97	0.50[a]
BOVINE0001S00026610	ANKRD11	1.47	2.63[a]
BOVINE0001S00007162	APOBEC3A	1.04	2.26[a]
BOVINE0001S00002481	ATP5B	0.59	0.50[a]
BOVINE0001S00005002	C3	2.43[a]	2.35[c]
BOVINE0001S00004513	C4BPA	1.49	3.15[c]
BOVINE0001S00002673	CFB	4.90[b]	2.04[b]
BOVINE0001S00017664	CFI	3.35[b]	2.77[c]
BOVINE0001S00016446	CHI3L1	1.64	2.24[a]
BOVINE0001S00018071	CLCA1	5.43[c]	3.55[b]
BOVINE0001S00046459	CLDN2	7.82[a]	9.13[b]
BOVINE0001S00002060	COL1A1	1.17	2.38[a]
BOVINE0001S00018672	DNAJA2	0.77	0.33[a]
BOVINE0001S00008747	EGR1	1.15	2.02[a]
BOVINE0001S00009754	EIF4GI	2.12	2.30[a]
BOVINE0001S00033368	FAIM3	1.01	2.34[a]
BOVINE0001S00029681	FCGBP	8.12[b]	7.01[c]
BOVINE0001S00018349	FCGR2B	2.27[a]	1.62
BOVINE0001S00029419	FGA	0.88	0.50[a]
BOVINE0001S00022705	FN1	2.24	2.20[a]
BOVINE0001S00023000	GKN2	0.86	0.43[a]
BOVINE0001S00006091	GNLY	1.07	2.17[a]
BOVINE0001S00014384	GSN	0.72	2.17[b]
BOVINE0001S00026894	GSTA3	0.83	0.49[b]
BOVINE0001S00002330	HDLBP	1.92	2.19[a]
BOVINE0001S00019652	HLA-DRB3	1.25	0.48[a]
BOVINE0001S00014411	ID2	0.97	0.38[a]
BOVINE0001S00013038	IGLL1	1.69	4.46[c]
BOVINE0001S00015260	ITIH4	1.97	2.50[b]
BOVINE0001S00030786	ITLN2	4.78[a]	10.32[a]
BOVINE0001S00018989	KNG1	1.21	3.32[b]
BOVINE0001S00008522	LGALS15	1.55	4.62[a]
BOVINE0001S00004672	MED13L	0.79	0.50[a]
BOVINE0001S00019995	METTL7A	0.50[a]	0.62
BOVINE0001S00017977	MGP	0.48	0.42[a]
BOVINE0001S00006539	MMP1	2.55[a]	1.46
BOVINE0001S00021086	MMP13	2.51[a]	1.22
BOVINE0001S00020378	MMP3	3.87[b]	4.03[c]
BOVINE0001S00040625	MMP7	1.89	4.21[a]
BOVINE0001S00002999	MPTX	1.39	4.63[b]
BOVINE0001S00043579	MSMB	3.16	5.53[c]
BOVINE0001S00040421	MYH14	2.00[a]	2.02
BOVINE0001S00045757	NCF4	0.93	2.87[a]
BOVINE0001S00043599	PACAP	0.60	2.35[a]
BOVINE0001S00016772	PDK4	0.90	0.22[a]
BOVINE0001S00042144	PIGR	2.12	3.06[a]
BOVINE0001S00004013	PLA2G2A	1.60	0.49[a]
BOVINE0001S00030964	PLAUR	1.05	0.29[a]
BOVINE0001S00044574	RIPK4	1.13	0.47[a]
BOVINE0001S00034240	SERPINI1	0.80	0.41[a]
BOVINE0001S00007318	SFTPD	1.80	2.88[b]
BOVINE0001S00007680	SGK2	0.89	0.37[a]
BOVINE0001S00035129	SLC22A16	1.26	0.49[a]
BOVINE0001S00041314	SLC44A2	1.53	2.11[a]
BOVINE0001S00032320	SPINK1	0.89	0.43[a]
BOVINE0001S00039455	SPLUNC2B	0.24[a]	0.82
BOVINE0001S00010616	TCN1	1.35	3.37[a]
BOVINE0001S00003383	TFF3	2.78[b]	2.01[c]
BOVINE0001S00003478	TNC	2.01[a]	1.39
BOVINE0001S00040610	TNS4	1.68	2.14[b]
BOVINE0001S00038296	ZNF404	0.73	0.50[a]

Note: Number denotes fold change, which is expressed as 14 dpi vs. their respective controls (naïve control for the primary infection or drug-attenuated control for the repeat infection). The gene name (description) can be found in Supplementary Table 1.

[a] P value ≤ 0.05.
[b] P value ≤ 0.01.
[c] P value ≤ 0.001.

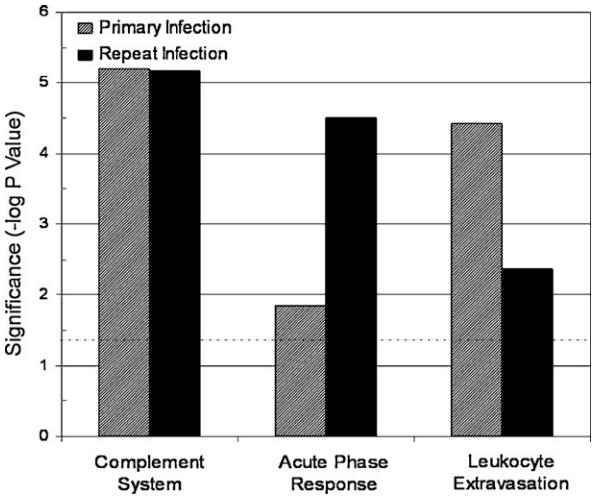

Fig. 1. Pathways impacted in the bovine abomasum during *Ostertagia ostertagi* infection based upon the level of significance. The dashed line represents a significance level at $P < 0.05$.

abomasal mucosa in both the primary and repeat infections (Table 3). C4 binding protein (C4BPA), a key regulator of the classical pathway of complement activation, was only markedly up-regulated during the repeat infection. C4A, one of the two genes encoding complement C4, was also up-regulated (though not statistically significant) during the repeat infection. Similarly, several genes belonging to acute phase responses, including fibrinogen α chain (FGA), fibronectin 1 (FN1) and inter-alpha (globulin) inhibitor H4 (ITIH4), were strongly induced during reinfection. The genes in leukocyte extravasation signaling impacted during both infections included MMPs, CLDN2 and neutrophil cytosolic factor 4 (NCF4).

3.3. Quantitative PCR

Expression of 14 genes at mRNA levels in the abomasal mucosa during both primary and repeat infections was examined using quantitative RT-PCR. As Table 3 shows, mRNA molecules of complement components, C3, factor B (CFB) and factor I (CFI), were continuously present at a low level in the normal uninfected tissue. Primary infection significantly induced the expression of these genes. All three genes were induced to a much greater extent during repeat infection (although from a lower level in the drug-attenuated controls) than during primary infection. For example, fold change for C3 mRNA by the repeat infection (vs. the drug-attenuated controls) was 45 compared to 15-fold induced by the primary infection (vs. their naïve controls). These results confirmed what was observed from the microarray data.

J chain (IGJ) plays a crucial role in the formation of immunologically important secretory immunoglobulins because this molecule is required for the formation of polymeric IgA and pentameric IgM, enabling their high affinity binding to polymeric Ig receptor (PIGR) for transepithelial transport and secretion. IGJ mRNA was up-regulated 63.8-fold during repeat infection, suggesting that immune animals may have developed an accelerated ability for the production of secretory IgA and IgM. Consistent with this, PIGR, a receptor for both polymeric IgA and IgM, as well as a specific IgM receptor, Fas apoptotic inhibitory molecule 3 (FAIM3), were strongly up-regulated during *O. ostertagi* infection. Quantitative PCR also confirmed the induction of MMP13 by the infection.

Expression of cytokines is largely cell-specific. The patterns obtained from draining lymph nodes are distinct from tissues at the site of infection. The cytokine-secreting cells may represent a tiny percentage of total cells in the abomasal mucosa. Therefore, a relatively low mRNA level of cytokines in the tissue, along with large variations in out-bred populations commonly used in challenge experiments and the small sample size, may, at least partially, explain inconsistent observations from published reports. In this study, the transcripts of IL-5 and IL-13 were barely detectable after 40 cycles of RT-PCR. TNFα mRNA was detectable but appeared not to be induced by the infection. IL-4 mRNA was strongly induced by the infection.

Table 3
Copy numbers of selected genes in the abomasal mucosa detected using quantitative PCR. The number of molecules (copy number) is presented as mean ± SEM.

Gene	Naïve control	Primary infection	Drug-attenuated	
			Control	Infection
C3	1613.3 ± 173.2	24533.3 ± 6809.3**	111.5 ± 34.5	5054.7 ± 4182.6
CFB	17350.0 ± 1003.7	165666.7 ± 21672.4***	11066.7 ± 3649.7	158200.0 ± 90984.5*
CFI	2006.7 ± 526.8	25283.3 ± 6224.3**	49.1 ± 8.3	2858.7 ± 2093.6
PIGR	3276.7 ± 1148.6	53733.3 ± 17986.0*	107.7 ± 9.5	4879.5 ± 4486.0
FAIM3	21.6 ± 8.2	107.8 ± 36.9	3.4 ± 4.5	32.6 ± 41.6
IGJ	40150.0 ± 9351.6	112583.3 ± 33683.3	9985.0 ± 3544.4	636933.3 ± 531715.4*
IL-4	5.6 ± 1.9	165.8 ± 50.5*	3.9 ± 0.23	54.0 ± 1.7***
IL-10	101.5 ± 15.7	91.0 ± 4.5	39.1 ± 6.3	293.3 ± 243.7
IFNG	389.2 ± 180.3	116.6 ± 28.2	207.1 ± 96.6	261.6 ± 178.7
MMP13	341.5 ± 90.0	5988.3 ± 1444.1**	334.7 ± 185.6	898.2 ± 439.2
IGLL1	182.4 ± 37.1	14870.0 ± 2153.1**	19.9 ± 9.6	241.3 ± 203.3

Note: (1). Copy numbers in 100 ng total RNA equivalents from the abomasal mucosa based on the standard curve approach. (2). Log-transformed copy numbers were analyzed using unpaired *t*-test between the infected animals and their respective controls (i.e., naïve Controls vs. the primary infected and the drug-attenuated controls vs. the re-infected).

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3.4. Western blot analysis

Expression of selected proteins in the abomasal mucosa during *O. ostertagi* infection is depicted in Fig. 2. Consistent with mRNA results obtained using quantitative PCR, IL-5 protein was not detectable, suggesting eosinophils may not be the predominant effector cells in the abomasal mucosa during *Ostertagia* infections. IL-1β protein expression was elevated 4.8-fold during the primary infection. Its elevated expression was maintained during the drug treatment and resting period (as in the drug-attenuated control) and induced additional 2.5-fold by the repeat infection. NCF4 expression at the protein level was also strongly up-regulated during infections. As shown in Fig. 2, NCF4 protein was ~2-fold higher in the abomasal mucosa during the primary infection compared to the naïve control. Similarly, IGLL1 (λ5) protein, which is required for rearrangement of the Ig κ light chain gene; and IGLL1 knockout mice showed severe impairment of B cell development from pro-B to immature B cell (IgM+) (Miyazaki et al., 1999), was also slightly stimulated in the abomasal mucosa by the infection.

In vivo activation and degradation of native complement C3 protein generate several C3 fragments, including C3b, iC3b, C3c and C3dg. The native C3 and C3c are not active in complement activation and regulation. Therefore, measuring activated C3 becomes of paramount importance in understanding the complement cascade. In this study, activated C3 was measured using a human monoclonal antibody I3/15, which recognizes a neoepitope that is not present in native C3 but expressed in C3b, iC3b as well as C3dg. The relative amount of activated C3 in the abomasal mucosa was expressed as C3b/iC3b/C3dg. Consistent with mRNA expression of native C3 detected using quantitative PCR, activated C3 was higher in the primary infected group compared to naïve controls. The repeat infection further stimulated activation of C3 (Fig. 3).

4. Discussion

Protective immunity to *O. ostertagi* infections in cattle develops very slowly. Resistance to reinfection manifests only after prolonged exposure. In an attempt to dissect mechanisms underlying protective immune responses, we developed two infection protocols, primary infection and drug-attenuated repeat infection. The goal was to obtain immune animals harboring significantly reduced numbers of parasites upon re-exposure, and then compare the transcriptomics characteristics of the abomasal mucosa between the two infected groups with their respective controls. Data on worm burdens from this experiment, as in many other similar challenge experiments conducted using out-bred populations, displayed a large variation. While L3 larvae used in both infection protocols were indeed from the same parasite population, the number of worms recovered from the primary infection was seemingly low. Miscounting the worms recovered during the experiment was possible. Other confounding factors could include infectivity of L3 larvae and differences in aging of calves between two infections. Most importantly, different levels of worm burdens could have a strong impact on

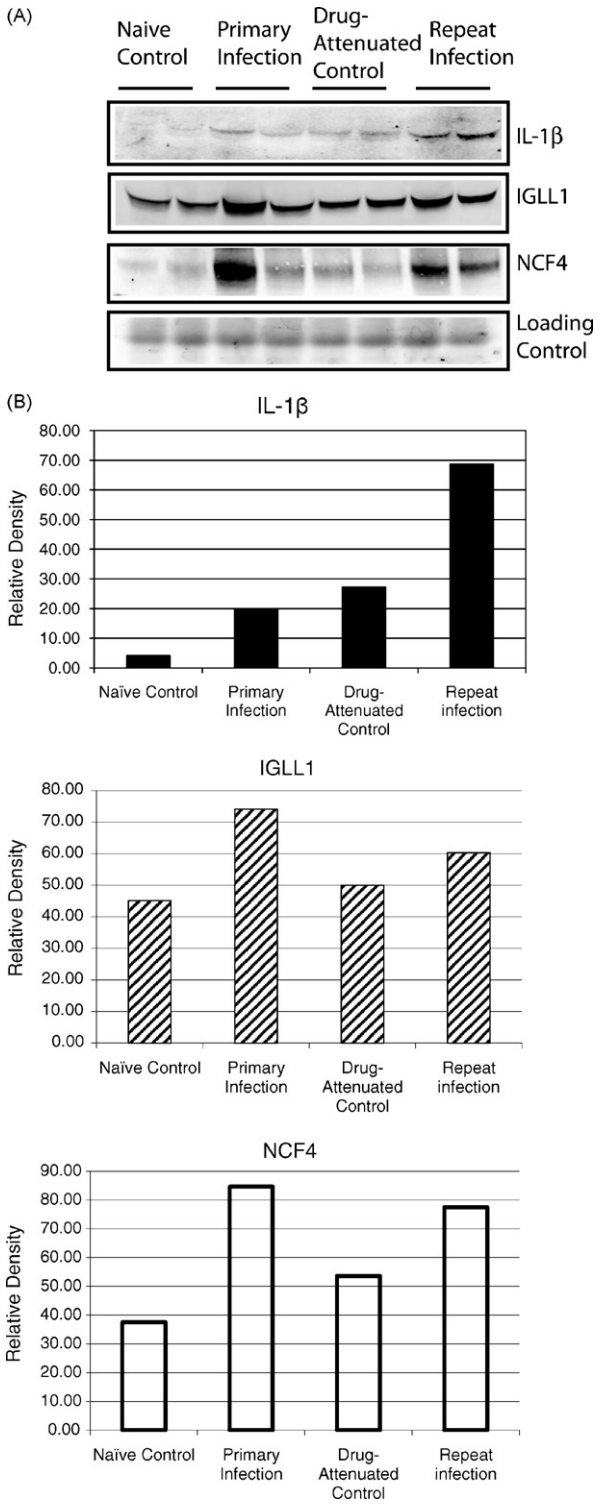

Fig. 2. Western blot analysis of select proteins in the bovine abomasum during primary infection and drug-attenuated reinfection for 14 days. (A) The Western blot image. (B) The relative density of the target protein.

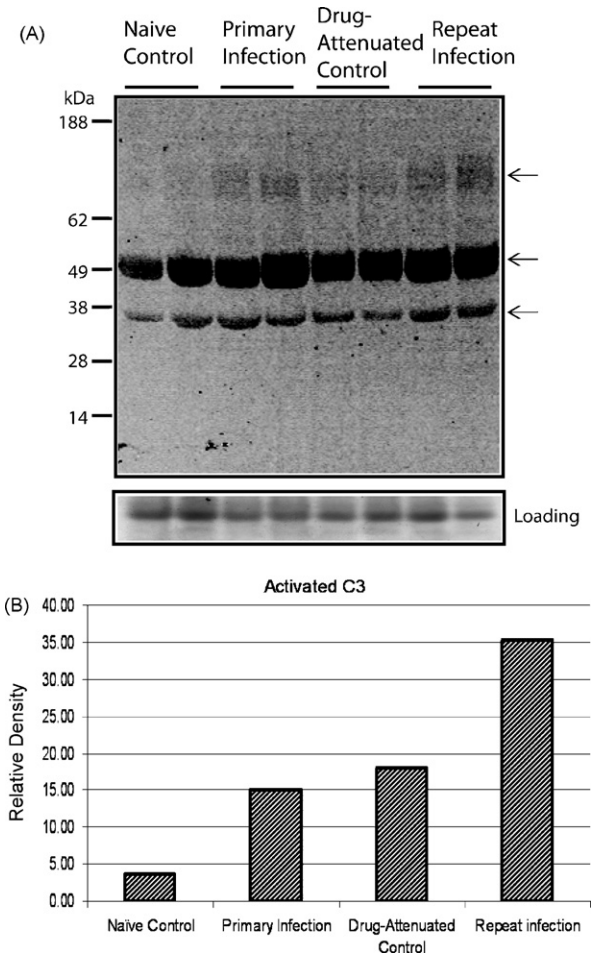

Fig. 3. Activated C3 in the bovine abomasum 14 days after *Ostertagia ostertagi* infection. The antibody used is a mouse monoclonal antibody I3/15 that recognizes a neoepitope expressed only in C3b, iC3b, and C3dg but not in native C3 (sc-47687) from Santa Cruz Biotechnology. The relative amount of activated C3 is expressed as C3b/iC3b/C3dg. (A) The Western blot image. (B) The relative density of the target protein.

gene expression. Therefore, caution is warranted in interpreting gene expression data. Nevertheless, our results on cytokine expression generally confirmed previously published reports that in the abomasal mucosa, mRNA of selected cytokines is generally detectable and *Ostertagia* infections in cattle elicit a Th2-like response. Interestingly, we found, as showed in Fig. 2, that at the site of infection (i.e., the abomasal mucosa), IL-1β protein was elevated in the abomasums during the primary infection and its elevated expression was well maintained in the drug-attenuated controls and further stimulated by the repeat infection. This cytokine may play an important role in regulating multiple signaling pathways.

Secretory immunoglobulins (SIg), such as IgA, provide adaptive immunologic protection of mucosal surfaces and ensure immune exclusion by serving as an external barrier trapped within mucus. In addition, SIg is able to neutralize intracellular microbial pathogens, such as viruses, directly within epithelial cells, as well as to bind to antigens and reduce their access to systemic circulation (Mazanec et al., 1993). Both polymeric IgA and pentameric IgM produced by plasma cells, which are located in the mucosal lamina propria, diffuse through the stroma and bind to PIGR for transepithelial transport. The complexes are then cleaved; and secretory IgA and IgM as well as secretory component (SC) are released into the lumen. Both J chain and PIGR are essential for SIg generation (Hendrickson et al., 1995). Only polymeric IgA and IgM containing J chain can bind to PIGR, which can mediate only one round of transepithelial transport of dimeric IgA. Therefore, a sustained increase in polymeric Ig transport requires an increased level of PIGR production by epithelial cells, thus making its production a rate-limiting step. PIGR synthesis is strongly regulated by cytokines, such as IL-1β and IL-4. PIGR knockout mice being more susceptible to infections (Tjarnlund et al., 2006). Similarly, J chain synthesis and secretion of IgM are differentially regulated by LPS and IL-5 (Randall et al., 1992), resulting in different ratios of two functional polymeric forms of IgM, hexameric and pentameric, which have different potency as ligands for complement activation via the classical pathway. Evidence suggests that antibody responses develop slowly against *O. ostertagi* antigens in cattle. In most infection protocols, the production of parasite-specific serum IgG, IgM and IgA is generally very weak (Klesius et al., 1986). Nevertheless, serum anti-*Ostertagi* IgA levels are significantly higher in infected cattle (Christensen et al., 1992). The IgG1, IgM, and IgA levels are significantly greater in the heavily infected animals. Total serum *O. ostertagi* antibodies in calves are also significantly elevated during a trickle infection (Forbes et al., 2009). In addition to infection doses, parasite development stages have an impact on antibody responses. Adult worms tend to cause marked increases in antibody levels in cattle (Entrocasso et al., 1986). A non-protective primary *O. ostertagi* infection in cattle caused an increase in both IgM+ and TcR1+ cells, a reduction in the percentage of T cells and IL-2, and an increase in the percentage of B cells, IL-4 and IL-10 (Canals et al., 1997). The increase in IgM+ (B cells) is consistent with an increase in IL-4. However, the role of antibodies, specifically local SIg, in resistance to *O. ostertagi* in cattle is not clear. Because *O. ostertagi* does not undergo systemic migration during its life cycle and is restricted to the abomasum, it is of critical importance to understand SIg in order to dissect mechanisms underlying the development of protective immunity. Nematode worms are generally too large to be phagocytosed by neutrophils. The most likely scenario is that infiltrated mast cells and basophils release granule contents upon activation, thereby creating a hostile environment for parasites and subsequently affecting the development and/or fecundity of parasites. A significant and negative correlation between the mucosal IgA levels and fecal egg counts and the number of eggs per female parasite, suggests that mucosal IgA may be associated with reduced *Ostertagia* fecundity (Claerebout and Vercruysse, 2000). An association between increased local IgA levels and decreased adult female worm length in sheep was also observed (Stear et al., 1995). Although reduced worm length can result from inhibited growth or selective expulsion of large worms, worm length is often an indicator of fecundity. These data suggest the local IgA response may be

an important effector mechanism in the cattle–*Ostertagia* interaction.

In the present study, we detected strong up-regulation of PIGR, IGJ and FAIM3 during infections. Over-expression of PIGR is suggested to be related with parasite resistance in Angus heifers (Li et al., 2007). Relatively higher IGJ mRNA levels were also detected in mesenteric lymph nodes in parasite resistant cattle compared to the susceptible animals (Araujo et al., 2009). Our observation that FAIM3, a protein may function as an uptake receptor for IgM-opsonized antigens by B cells (Shima et al., 2010), was up-regulated by the infection was intriguing. This receptor is expressed predominantly on B-lineage cells with its mRNA observed from pre-B cell stage and maintained thereafter during B cell development. Secretory IgA as well IgM-antigen complexes could serve as powerful inducers of complement activation.

The complement system is an essential part of innate immunity functioning as a powerful defense mechanism in eliminating invading pathogens and mediating inflammation. Complement also plays a regulatory role in controlling adaptive immune responses by building a bridge between innate and adaptive immunity. Unlike systemic complement proteins that are mainly produced in the liver, many cells in extrahepatic tissues, such as gut epithelial cells, endothelial cells, macrophages and neutrophils, are also capable of synthesizing complement components. Mounting evidence suggests the presence of functionally active complement components in many tissues under both normal and disease conditions. Locally synthesized complement components have been implicated in many diseases (Barnum, 1995; Sacks and Zhou, 2003; Chen et al., 2010). For example, up-regulation of mRNA for C1q, C3 and C4 and activation of the classical pathway are observed in the brain of patients with Alzherimer's disease (Yasojima et al., 1999).

Factor B (CFB) plays an important role in the alternative pathway of the complement cascade. CFB knockout mice have a significantly lower number of infiltrated neutrophils, the first and dominant leukocytes that migrate from the circulation to the site of infection (Mihai et al., 2007). Strong up-regulation of C3 and CFB as well as factor I (CFI) in the abomasal mucosa during the primary *Ostertagia* infection suggested that the alternative pathway may be activated early during infection and was readily re-activated, to a greater extent, during repeat infections. The alternative pathway provides a rapid, antibody-independent route of complement activation and could play a pivotal role in immune responses against *O. ostertagi* infection in cattle. Its activation is initiated by factors such as IgA, zymosan and LPS and is independent of immune complexes. The anaphylotoxin generated during the complement activation may play an important role in mediating inflammation, which represents an indispensible part of host responses. It is possible that a third pathway, the lectin pathway of the complement cascade, was also activated during *Ostertagia* infection. Our data showed that ITLN2 and several C-type lectins, such as collectin 11 (COLEC11), cattle-specific collectin-46 and conglutinin, as well as galectins were up-regulated in the abomasal mucosa during infections. Ficolin B and MBL were also slightly induced by infections (though not statistically significant). The elevated expression of ITLN2, which is regulated by the Th2 cytokine IL-4 (French et al., 2007), is also observed in the sheep abomasums after *Teladorsagia circumcincta* infection and *Dictyocaulus filaria* natural infection (French et al., 2009). The specific expression pattern of ITLN2, along with the fact that its presence in the genome of nematode-resistant mouse strain BALB/c and its deletion from that of susceptible strain C57BL/10, suggests that this gene may serve a protective role in the innate immune response to parasite infection (Pemberton et al., 2004). Over-regulation of ITLN2 was also observed in the sheep abomasums during *Haemonchus contortus* infection (Rowe et al., 2009). Collectin-46, one of the three bovidae-specific collectins, has been suggested to provide the first line of defense against pathogens without eliciting general inflammatory reaction (Hansen et al., 2002). Galectins, on the other hand, serve as receptors for pathogen-associated molecule patterns (PAMPs) and potential damage-associated molecular patterns (DAMPs), recognizing carbohydrate moieties on the cell surfaces of parasites, activating immune cells, such as neutrophils, eosinophils, mast cells and basophils, and participating in cytotoxicity, as well as promoting reconstruction of damaged tissues (Sato et al., 2009). In addition, galectins may bind to galactose residues on IgA molecules, playing an important role in modulating host responses to *O. ostertagi* infections in cattle. Together, these molecules may trigger activation of immune responses by initiating activation of the lectin pathway through attached MBL-associated serine protease (MASP).

In bovine abomasal mucosa, C4BPA mRNA was strongly up-regulated only during the repeat infection while C4A was slightly up-regulated, suggesting that the classical pathway, which is generally activated by immune complexes such as IgM and IgG and by molecules such as C-reactive protein via binding to C1q, could also be involved in protective immunity. Previously published reports plus data from this study clearly showed an increased production of these immunoglobulins during the repeat infection. Our data showed that mRNA levels of complement regulators, such as decay accelerating factor (CD55) and complement regulatory protein (CD59) were also slightly reduced by reinfection, in contrast to a strong induction of C3 and CFB mRNA molecules. This slight reduction of mRNA levels of complement regulators could permit enhanced complement activation during reinfection.

After binding to their targets, secretory IgA and PAMP-recognizing collectins (such as ficolins, MBL, collectin-46 and conglutinin) trigger a cascade of events, including activation of the complement system and recruitment of leukocytes to the site of infection (the abomasal mucosa). The amplification loop resulting from activation of the alternative pathway could allow for a sustained elevation of inflammatory cytokines, which along with reactive oxygen species and nitride oxide as well as proteases released by infiltrates, lead to amplification of local inflammation, thus creating a hostile environment for worms and providing an efficient containment for parasites. These factors may be instrumental in the development of protective immunity.

Epithelial integrity of abomasal mucosa is of critical importance in maintaining proper physiological functions of the abomasum. Ingested *Ostertagia* larvae invade and then develop within gastric glands, resulting in considerable damage to the abomasal mucosa when they exit the glands as adults. It is also conceivable that mucosal microcirculation would be compromised due to gastric gland damage during larvae development within the glands. Consequently, oxygen supply would be limited. TFFs are often strongly induced after epithelial damage (Cook et al., 1997). TFFs promote mucosal defense and tissue restitution, as well as long-term tissue repair in the GI tract by stimulating cell migration to re-establish mucosal continuity; inhibiting apoptosis; and enhancing mucosal properties (Taupin et al., 2000). For example, among three TFFs, only TFF3 is essential for epithelial restitution (Mashimo et al., 1996). TFF3 knockout mice display increased intestinal apoptosis and over-expression of TFF3 in a gastric cell line is antiapoptotic (Taupin et al., 2000). Evidence suggests that trefoil peptides and mucins co-exist in most mucus-producing cells in the GI tract. For example, MUC2 and TFF3 are co-localized throughout the large and small bowel mucosa. TFF peptides interact with mucin to increase viscosity and elasticity and induce mucin solution transformation into a gel-like structure (Thim et al., 2002), enabling protective and healing functions. One of the TFF binding proteins, gastrokine 2 (GKN2), is down-regulated by pro-inflammatory cytokines such as IL-1β (Baus-Loncar et al., 2007). The observation that the regulation of GKN2 often parallels that of TFF genes leads to a hypothesis that together they may play an important role in maintaining the homeostasis of the GI tract. In this study, TFF3 was strongly up-regulated and GSk2 was significantly down-regulated by infection. In addition, glucosaminyl (N-acetyl) transferase 3, mucin type (GCNT3), which catalyzes one of the key rate-limiting steps in mucin biosynthesis (Li et al., 2009), was up-regulated in the bovine abomasum during *Ostertagia* infections (Li and Gasbarre, 2010). Together, these results suggested enhanced tissue repair and mucin secretion may contribute directly to protective immunity.

Extracellular matrix (ECM) is a dynamic structure consisting of many structural and non-structural proteins, including various collagens, fibrillin, and glycoproteins, such as fibronectin, and receptors. ECM is able to direct or modulate cells to change their metabolism through the regulation of certain genes at the mRNA level. EMC remodeling is required for many normal and pathological processes, such as blood vessel formation and tissue repair, including the degradation of pre-existing ECM molecules, as well as the neosynthesis of ECM components. MMPs play a pivotal role in ECM remodeling. Besides their ability as proteases to guide remodeling by degrading ECM components and releasing matrix-bound growth factors, MMPs also function as multifaceted pro- and anti-angiogenic factors in angiogenesis, an integral part of tissue repair. Diverse functions of MMPs are also reflected by their ability to modify non-ECM proteins. For example, matrilysin (MMP7) is involved in shredding or release of several important signaling molecules, such as IGFBP, EGF, Fas L and TNFα, thereby regulating numerous biological processes, such as angiogenesis, inflammation, and apoptosis (Ii et al., 2006).

In this study, elevated mRNA levels of ECM components, including COL1A1, FN1, MMPs and TNC were observed during repeat infection, while fibrinogen (FGA) and uPA receptor (PLAUR) were down-regulated. Kininogen 1 (KNG1) plays an important role in inflammation and promoting neovascularization and is usually co-expressed with VEGF in inflamed eyes (Zipplies et al., 2010). Up-regulation of KNG1 during repeat infection in bovine abomasal mucosa suggested that it may be involved in tissue repair and angiogenesis. Together, altered expression profiles of selected ECM components during *Ostertagia* infections may be an indication of possible accelerated abilities for tissue repair in immune animals.

In conclusion, local synthesis and deposition of C3 protein in the abomasal mucosa could be a hallmark of *O. ostertagi* infection in cattle. Our findings provide the first evidence of the involvement of complement activation in the development of protective immunity against *O. ostertagi* infections in cattle. Future work will focus on examination of the relative contribution of all three complement activation pathways. Enhanced abilities for tissue repair may also contribute to the development of long-term protective immunity. Strong induction of GCNT3, a rate-limiting enzyme in mucin production, may promote restoration of mucin-secreting function of mucosal layers and thus enhance its barrier function. Increased synthesis of collectins and galectins may be necessary steps in tissue repairs due to their functions as DAMP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.08.037.

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